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Melatonin Synthesis Enzymes in *Macaca mulatta*: Focus on Arylalkylamine *N*-Acetyltransferase (EC 2.3.1.87)

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Arylalkylamine *N*-acetyltransferase (AANAT; serotonin *N*-acetyltransferase, EC 2.3.1.87) plays a unique transduction role in vertebrate physiology as the key interface between melatonin production and regulatory mechanisms. Circulating melatonin is elevated at night in all vertebrates, because AANAT activity increases in the pineal gland in response to signals from the circadian clock. Circadian regulation of melatonin synthesis is implicated in a variety of human problems, including jet lag, shift work, insomnia, and abnormal activity rhythms in blind persons. In this report AANAT was studied in the rhesus macaque to better understand human melatonin regulation. AANAT mRNA is abundant in the pineal gland and retina, but not elsewhere; AANAT mRNA is uniformly distributed in the pineal gland, but is limited primarily to the photoreceptor outer segments in the retina. Day and night levels of pineal and retinal AANAT mRNA are similar. In contrast, AANAT activity and protein increase more than 4-fold at night in both tissues. The activity of hydroxyindole-*O*-methyltransferase, the last enzyme in melatonin synthesis, is tonically high in the pineal gland, but is nearly undetectable in the retina; hydroxyindole *O*-methyltransferase mRNA levels exhibited a similar pattern. This supports the view that the source of circulating melatonin in primates is the pineal gland. The discovery in this study that rhesus pineal AANAT mRNA is high at all times is of special importance because it shows that posttranscriptional control of this enzyme plays a dominant role in regulating melatonin synthesis. (*J Clin Endocrinol Metab* 87: 4699–4706, 2002)

A CONSERVED FEATURE of vertebrate physiology is the nocturnal increase in circulating levels of the pineal hormone, melatonin, which functions to mediate many aspects of vertebrate circadian and seasonal physiology (1). Melatonin modulates sleep and circadian rhythmicity in humans and may play a therapeutic role in abrogating the negative effects of abnormal rhythms in situations such as jet lag, shift work, and delayed sleep phase insomnia (2). Likewise, manipulation of melatonin levels may help alleviate rhythm problems associated with seasonal affective disorder, blindness, and old age (2).

The melatonin rhythm is generated by the circadian clock located in the suprachiasmatic nuclei, which, in turn, stimulates the pineal gland through a neural circuit that passes through central and peripheral structures. Suprachiasmatic nuclei stimulation at night results in the release of norepinephrine from nerve terminals in the pineal gland. Norepinephrine acts through a dual α-/β-adrenergic$\rightarrow$cAMP/Ca^{2+} system to increase the activity of the penultimate enzyme in melatonin synthesis, arylalkylamine *N*-acetyltransferase (AANAT; serotonin *N*-acetyltransferase, EC 2.3.1.87) (3). A key event is cAMP-dependent phosphorylation of AANAT at one of two cAMP-dependent protein kinase (PKA) sites, which results in binding of the enzyme to 14-3-3 protein, leading to activation and probable stabilization of the enzyme (4, 5).

AANAT converts serotonin to *N*-acetylserotonin, which is then converted to melatonin by hydroxyindole *O*-methyltransferase (HIOMT; EC 2.1.1.4), which does not play a regulatory role in rapid changes in melatonin production. In contrast, the essential and unique regulatory role of AANAT in melatonin synthesis has made it a focal interest in pineal and circadian biology.

Comparative analysis of AANAT has revealed conserved similarities and interesting differences in the patterns of distribution and regulation, making it somewhat difficult to predict how the activity of human AANAT (hAANAT) is controlled. For example, AANAT in all species contains the PKA/14-3-3 binding motif; also, day/night and light-induced changes in AANAT activity are paralleled by changes in AANAT protein. However, the role of transcription in AANAT regulation differs markedly. In some species there is a large (up to 200-fold) nocturnal increase in AANAT mRNA in some species (*e.g.* rodents and chickens), whereas in others AANAT mRNA undergoes less than a 1.5-fold increase at night (*e.g.* ungulates and trout) (3). These differences appear to determine whether melatonin synthesis can occur immediately after lights off at night, as is the case in humans, primates, and ungulates (2, 6, 7), or if it is delayed, as is the case in rodents (8), by determining when AANAT translation occurs.

The relative level of AANAT expression among tissues also exhibits interesting species differences. AANAT mRNA expression in all vertebrate pineal glands is high. In the rat, AANAT expression is highly restricted to the pineal gland; retinal levels are approximately 250-fold lower than pineal levels, and AANAT mRNA is not detectable elsewhere in the brain, except by Southern blotting of RT-PCR products (8–

Abbreviations: ANAT, Arylamine *N*-acetyltransferase; AANAT, arylalkylamine *N*-acetyltransferase; h, human; HIOMT, hydroxyindole *O*-methyltransferase; ORF open reading frame; PKA, cAMP-dependent protein kinase.

10). In contrast, retinal AANAT mRNA levels in sheep and humans are about 25% of those in the pineal gland (11, 12), and weak expression of AANAT mRNA is detectable by Northern blot analysis in the pituitary and selected brain regions (3, 13).

Although AANAT has been studied in human tissues (12), it is difficult to assess whether day/night rhythms exist in the pineal gland and retina because of practical limitations and associated uncontrolled tissue degradation. Accordingly, we have turned to the rhesus macaque as a model for understanding human regulation of melatonin synthesis because of their evolutionary relationship and because previous reports have indicated that circulating melatonin is regulated in both in a similar manner, *i.e.* there is an immediate increase in circulating melatonin at the onset of darkness at night (2, 7), and dim white light suppresses melatonin production (1, 14, 15).[1] The results of these efforts are presented here.

Materials and Methods

Animals and tissue preparation

Adult rhesus macaques (*Macaca mulatta*) were obtained through the Veterinary Resources Program Primate Recycle Program of NIH. All protocols and handling of the animals conformed to the Guidelines for Care and Use of Laboratory Animals (NIH Publication 80-23; Protocol 99-012). Animals were housed under a 12-h light, 12-h dark lighting regimen (lights on at 0600 h) in NIH facilities for at least 6 wk. The light level in the cages was approximately 325 Lux. "Day" animals were killed between 1030–1300 h; "night" animals were killed between 2200–0100 h. Before transport to the procedure area, animals were anesthetized (ketamine, 0.1 ml/kg body weight), and a catheter was placed in the saphenous vein. Blood (~3 ml) was drawn into a nonheparinized tube via the catheter immediately before dissection; animals were killed using Beuthanasia D (Schering-Plough, Union, NJ). For animals sampled at night, anesthesia was administered, and a blindfold was applied under dim red light, after which catheter placement and euthanasia took place under dim white light; the blindfold was removed only after decapitation. Tissues were removed and placed on dry ice.

Tissues were homogenized as required for specific procedures (see below and figure legends). Homogenates were centrifuged (15,000 × *g*, 15 min, 4 C), and supernatants were used for Western blot analysis and enzyme assays.

Cloning of rhesus monkey AANAT (rhAANAT) and rhesus monkey (rhHIOMT) cDNA

Clones of rhAANAT and rhHIOMT were obtained by PCR from pineal cDNA. Solid phase cDNA synthesis was performed on magnetic oligo(deoxythymidine) beads (Dynal, Lake Success, NY) using 5 μg total RNA as previously described (16). The full open reading frame (ORF) of AANAT was amplified using primers M02L (5′-GGTATCCTCTC-CACAGTCCAG) and M03R (5′-GCCGTGAGAAGCCACCTTATC). Transcripts were amplified using a high fidelity *Taq* polymerase (Takara ExTaq, Intergen, Purchase, NY) and were cloned into pGEM-T (Promega Corp., Madison, WI; rhAANAT clone MN4-1).[2] The full ORF of rhHIOMT was amplified using primers HH01L (5′-TCCTTGAAG-CAAGCGCTCC) and HH02R (5′-GTGCTTTGACGTTAGTTCCAGG). The PCR employed *Pfu* polymerase (Stratagene, La Jolla, CA), and products were cloned into pCR-Zero-Blunt-TOPO (Invitrogen, Carlsbad, CA; rhHIOMT clone MH11B).[2] For both rhAANAT and rhHIOMT, clones from three independent PCR reactions were sequenced and were found to be identical.

Northern blot analysis

Total RNA was extracted by a guanidine HCl/phenol method (TRIzol, Invitrogen), separated on a 1.5% agarose/0.7 M formaldehyde gel, transferred to a charged nylon membrane by passive capillary transfer, and immobilized by UV cross-linking. Blots were hybridized at 68 C for 1.5 h in QuikHyb buffer (Stratagene, La Jolla, CA). The final wash was in 0.1× standard saline citrate containing 0.1% sodium dodecyl sulfate at 60 C for 15 min. Blots were imaged and quantitated using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

Blots were probed sequentially using a full-length rhAANAT cDNA clone (MN4-1, see above), a full-length rhHIOMT cDNA clone (MH11B, see above), and a human glycerol-3-phosphate dehydrogenase probe (CLONTECH Laboratories, Inc., Palo Alto, CA), which was used to monitor RNA quality and loading. All hybridization probes were ^{32}P labeled by random priming. Transcript sizes were estimated by comparison with RNA markers (Roche, Indianapolis, IN).

Enzyme activity assays

Homogenates of pineal, retina, and pituitary were assayed for AANAT activity. Briefly, homogenates were incubated at 37 C (20 min) with [^{3}H]acetyl-coenzyme A (0.5 mM; Calbiochem, San Diego, CA; final specific activity, 4.2 Ci/mol; Perkin-Elmer Life Sciences, Wellesley, MA) and tryptamine (1 mM; RBI, Natick, MA) in 100 mM phosphate buffer, pH 6.8, as previously described (17). Homogenates were also assayed for arylamine *N*-acetyltransferase (ANAT; EC 2.3.1.5) activity in parallel under the same conditions using phenetidine (1 mM; City Chemical, New York, NY) in place of tryptamine (18). ANAT was measured because it weakly acetylates tryptamine, and changes in it might contribute to changes in apparent AANAT activity.

HIOMT activity was measured using the standard assay as previously described (19). Briefly, homogenates were incubated for 30 min at 37 C with [^{3}H]*S*-adenosyl-methionine (0.1 mM; Sigma, St. Louis, MO; final specific activity, 100 Ci/mol; Amersham Pharmacia Biotech, Piscataway, NJ) and *N*-acetylserotonin (1 mM; Sigma). The [^{3}H]melatonin formed was extracted into chloroform, which was dried, and radioactivity was determined using a scintillation counter.

Western blot analysis

Tissues were homogenized as described above. Total cellular proteins were separated on a 14% precast SDS-PAGE gel (Novex, Invitrogen). Each gel contained a lane loaded with 2 μl *in vitro* translated rhAANAT (see below), which was included for normalization. Proteins were transferred to polyvinylidene difluoride membranes and immunodetected as previously described (20). Total AANAT protein was immunodetected using immunopurified antiserum (3236) raised against a human peptide $hAANAT_{1–26}$ (Fig. 1) (20); this antiserum detects rhAANAT and hAANAT proteins with similar sensitivity. The phosphorylation state of the N-terminal PKA site was determined using immunopurified antiserum (3352) that had been raised against a rat AANAT phosphopeptide (phospho-$rAANAT_{22–37}$) (4).

In vitro translation of rhAANAT

rhAANAT was transcribed and translated using a rabbit reticulocyte lysate according to the manufacturer's instructions (TnT Quick Coupled Transcription/Translation system, Promega Corp.). The transcribed plasmid (MN5-7) contained the full-length AANAT ORF. Plasmid MN5-7 was derived from clone MN4-1 by removing two ATG start codons from the 5′-untranslated region. After 90 min of incubation, the reaction acetylated 1 mM tryptamine at a rate of 1.1 nmol/h·μl in the radiometric AANAT assay described above.

Analysis of serum melatonin

Blood was kept on ice for 1 h, then centrifuged (1500 × *g*, 15 min, 4 C). The serum was removed, placed on dry ice, and stored at –80 C until assayed for melatonin by RIA (6, 20).

[1] It had originally been thought that "man and monkey are not the same" with regard to the intensity of light required to suppress nocturnal melatonin levels (14, 36); however, this view is incorrect: human serum melatonin levels can be rapidly suppressed at night by low levels of light (15, 37).

[2] These sequence data have been submitted to the GenBank database under accession numbers AY069924 (rhHIOMT) and U46661 (rhAANAT); the rhAANAT amino acid sequence has been published (3).

AANAT

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                                        PKA/14-3-3
Rhesus    1 MSTQSTHPPKPEAPRLPPAIS---SCQRRHTLPASEFRCLTPEDAVSAFEIEREAFISVLGVC
Human     1 .......L.........G.PESP.....................................
                                  ****************

Rhesus   61 PLYLDEIRHFLTLCPELSLGWFEEGCLVAFIIGSLWDKDRLMQESLTMHRPGGHIAHLHVLAV
Human    64 ....................................E........L..S............

Rhesus  124 HCAFRQQGRGPILLWRYLHHLGSQPAVHRAALMCEDALVPFYERFGFHAMGPCAITVGSLSFT
Human   127 .R.........................R.................S...V..........T.M

                          PKA
Rhesus  187 ELHCSLQGHPFLRRNSGC
Human   190 ......R.....____..
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HIOMT

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Rhesus    1 MGSSGDDGYRLLNEYTNGFMVSQVLFAACELGVFDLLAEAPGPLDVAAVAAGVEASSHGT
Human     1 ....E.QA.....D.A.....................................R..A...

Rhesus   61 ELLLDTCVSLKLLKVETRAGKAFYQNTELSSAYLTRVSPTSQCNLLKYMGRTSYGCWGHL
Human    61 .....I............G.....R......D...T.......SM.........R.....

Rhesus  121 ADAVREGKNQYLQTFGVPAEDLFKAIYRSEGERLQFMQALQEVWSVNGRSVLTAFDLSGF
Human   121 .......R....E.......E..T................................V.

Rhesus  181 PLMCDLGGGPGALAKECLSLYPGCKVTVFDVPEVVRTAKQHFSFPEEEEIHLQEGDFFKD
Human   181 .........A.......M.......I....I....W........Q...Q.DF........

Rhesus  241 PLPEADLYILARILHDWADGKCSHLLERVYHTCKPGGGILVIESLLDEDRRGPLLTQLYS
Human   241 ............V..............I.............................

Rhesus  301 LNMLVQTEGQERTPTHYHMLLSSAGFRDFQFKKTGAIYDAILVRK
Human   301 .....................................A..
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FIG. 1. Comparison of rhesus monkey and human AANAT and HIOMT amino acid sequences. GenBank accession numbers are: AANAT: rhesus monkey, U46661; human, U40347; HIOMT: rhesus monkey AY069924; human U11098. Only nonconserved residues are shown for the human sequence. The peptide used to generate antiserum 3236 is *single underlined*; the peptide used for antiserum 3352 is *underlined with asterisks*. The N-terminal PKA/14-3-3 motif is *boxed*; the C-terminal PKA site is *double underlined*.

In situ hybridization histochemistry

Pineal glands, whole eyes, pituitaries, and brain regions were examined. Fresh-frozen sections (12 μm) were processed using ^{35}S-labeled riboprobes (21). Riboprobes corresponded to nucleotides 1–434 of monkey AANAT (GenBank accession no. U46661). Slides were dipped in Ilford K.5D nuclear emulsion (Ilford, Paramus, NJ) containing 300 mM ammonium acetate and exposed for up to 4 months before developing and counterstaining with toluidine blue.

Statistical analysis

The significance of differences between day and night values in Table 1 was tested by t test, with $P < 0.05$ considered significant (22). The degree of correlation between the level AANAT protein and AANAT activity was calculated as the coefficient of determination from linear regression analysis (22).

Results

Deduced rhAANAT and rhHIOMT protein sequences

The deduced amino acid sequences of rhAANAT and rhHIOMT were found to be 94% and 90% identical to those of the human genes, respectively (Fig. 1). Examination of the rhAANAT sequences indicates that the critical regulatory sequences are conserved, including the N-terminal PKA/14-3-3 motif (rhAANAT$_{25–30}$) and the C-terminal PKA site (rhAANAT$_{199–202}$).

Tissue distribution of rhAANAT and rhHIOMT mRNA

Northern blot analysis of selected rhesus tissues indicated that rhAANAT mRNA (1.1 kb) is abundant in the pineal gland and retina (Fig. 2). The ratio of retinal to pineal rhAANAT mRNA in each animal (per microgram of total RNA) was 2.1 ± 0.4 (n = 5; Table 1). Expression in the pituitary and testes was approximately 1/100th of pineal levels; rhAANAT mRNA was not detected in other tissues examined.

rhHIOMT mRNA was abundant in the pineal gland; transcripts of two sizes were detected (1.3 and 2.8 kb). In contrast, rhHIOMT mRNA was undetectable in about 50% of the retinas examined and was present in others at variable levels (ranging from 1/10th to 1/100th of that in the pineal); it was not detected in other tissues examined.

Analysis of day/night rhythms of plasma melatonin and tissue rhAANAT and rhHIOMT mRNA, protein, and activity

Melatonin. The night/day plasma melatonin ratio in animals used in this study was 4.3-fold, consistent with previous reports (7, 23, 24), providing evidence of normal circadian physiology. AANAT mRNA was detected at high levels in the pineal gland and retina in both day and in night samples (Fig. 2). A day/night rhythm in AANAT mRNA was not detected (Table 1). In contrast to the arrhythmic pattern of AANAT mRNA, a large rhythm in both AANAT activity and AANAT immunoreactive protein was evident, with night values approximately 10–fold greater than day values in the pineal; the night/day ratio of AANAT activity and protein

TABLE 1. Day and night AANAT, ANAT, and HIOMT activities and AANAT and HIOMT mRNA levels in the pineal gland, retina, and pituitary gland

Tissue	Sample time	Enzyme activity (nmol/h/mg protein)		mRNA (normalized)	
		AANAT	ANAT	AANAT	HIOMT
Experiment 1					
Pineal	Day	2.7 ± 1.3	21.0 ± 4.7	0.88 ± 0.14	0.81 ± 0.16
	Night	63.7 ± 12.1[a]	24.8 ± 5.8	1.12 ± 0.27	1.19 ± 0.14
Retina	Day	4.7 ± 0.3	24.4 ± 4.8	0.99 ± 0.12	Trace
	Night	25.5 ± 1.5[a]	31.7 ± 6.6	1.01 ± 0.08	Trace
Pituitary	Day	0.07 ± 0.025	51.8 ± 12.9	Trace	n.d.
	Night	0.09 ± 0.035	89.3 ± 8.2[a]	Trace	n.d.
Experiment 2		AANAT	HIOMT		
Pineal	Day	6.3 ± 1.7	9.0 ± 1.3	–	–
	Night	25.7 ± 3.7[a]	9.4 ± 1.9	–	–
Retina	Day	1.6 ± 0.5	n.d.	–	–
	Night	11.4 ± 0.9[a]	n.d.	–	–

In experiment 1, pineal glands, retinas, and pituitaries were removed from the same set of animals sacrificed at mid-day (n = 5) or midnight (n = 5). Tissues were homogenized in 50 mM phosphate buffer (pH 7.9) containing 1 mM dithiothreitol, protease inhibitors (0.5 mM *p*-aminoethylbenzenesulfonyl fluoride, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 1.0 μg/ml aprotinin, and 40 μg/ml bestatin, and an RNAse inhibitor (RNAsin, 200 U/ml; Promega, Madison, WI). After homogenization, RNA was immediately extracted from an aliquot and analyzed by Northern blot, as described in *Materials and Methods*. The remainder was frozen and stored at −80 C until enzyme assays were performed, as described in *Materials and Methods*. Northern blot data were normalized to G3PDH and expressed relative to the mean of all values within each tissue. In experiment 2, pineal glands and retinas were removed from another set of animals sacrificed at mid-day (n = 4) or midnight (n = 4). Tissues were homogenized in 50 mM phosphate buffer (pH 7.9) containing 1 mM dithiotheitol and protease inhibitors (Complete inhibitor cocktail, 1 tablet/50 ml, Roche, Indianapolis, IN). Homogenates were assayed immediately, or stored at −80 C until used. Differences in AANAT activity between experiments 1 and 2 may be due to differences in homogenization buffers and handling. Data are the mean ± SEM. n.d., Not detected.

[a] Significant increase at night ($P < 0.05$).

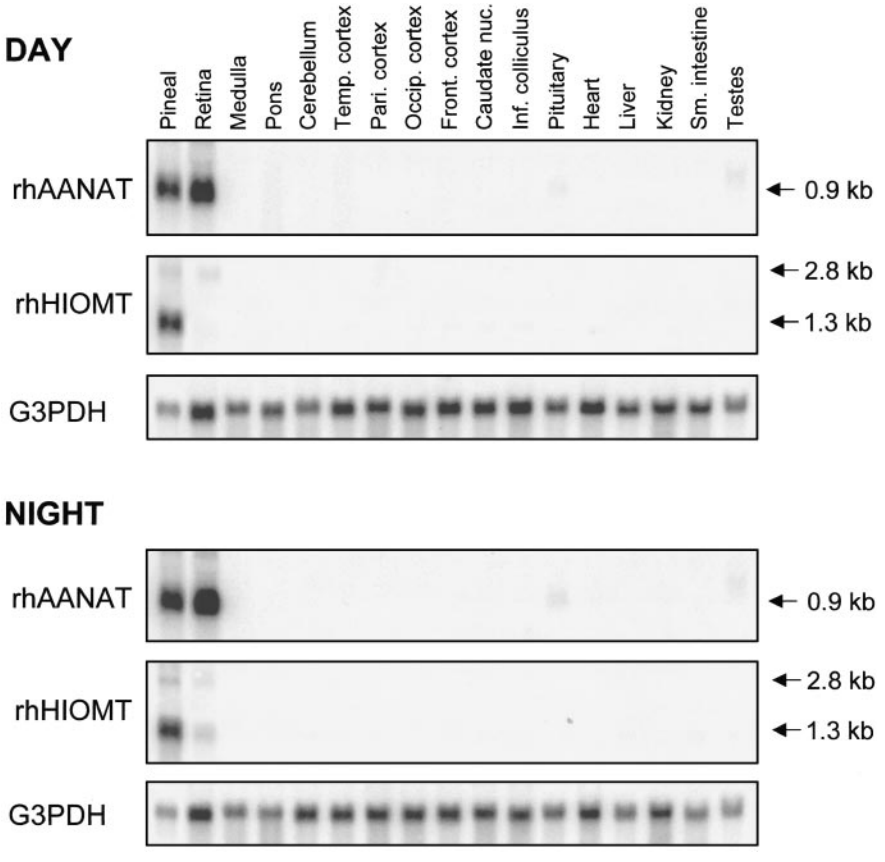

FIG. 2. Northern blot analysis of rhAANAT and rhHIOMT mRNA tissue distribution. Each lane contains 10 μg (pineal gland) or 30 μg total RNA (day or night). Tissues on each blot were obtained from a single animal. The blots were stripped and probed sequentially for rhAANAT, rhHIOMT, and glycerol-3-phosphate dehydrogenase. Temp., Temporal; Occip., occipital; Front., frontal; nuc., nucleus; Inf., inferior; Sm., small. For details of tissue preparation, see *Materials and Methods*.

in the retina was 5-fold (Fig. 3 and Table 1). The magnitude of the day/night difference is a minimum estimate; a more detailed sampling time may have determined the actual minima and maxima of the rhythms. The shape of the AANAT cycle in the rhesus macaque is likely to be similar to that of melatonin in the cerebrospinal fluid and blood (7), because of the close correlation among AANAT protein, AANAT activity, and circulating melatonin in rats, sheep, and other

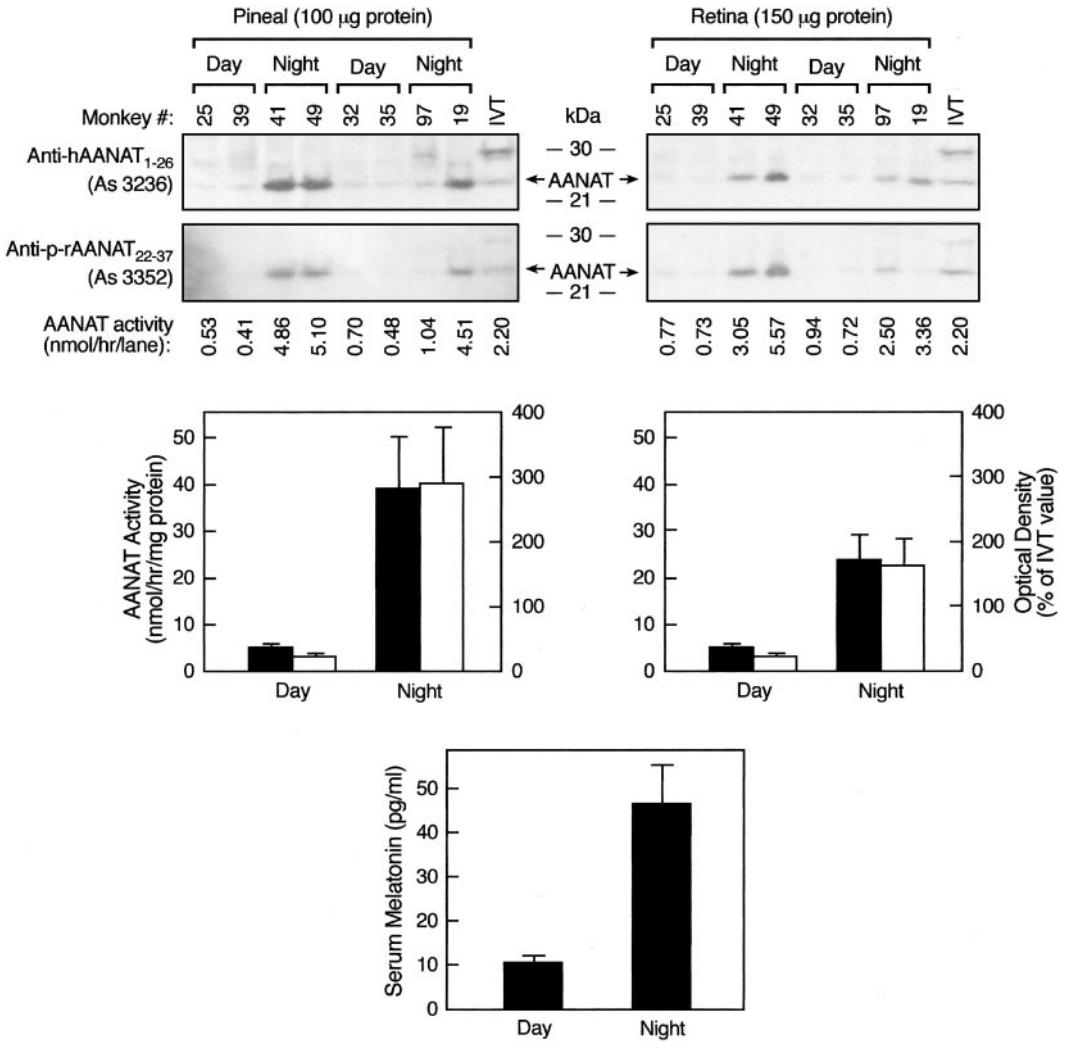

FIG. 3. Day/night rhythms in pineal and retinal rhAANAT protein and activity, and serum melatonin. Pineal glands and retinas were obtained from the same set of animals killed at midday (n = 4) or midnight (n = 4). Tissues were homogenized in 100 mM phosphate buffer, pH 6.8, containing 1 mM dithiothreitol, protease inhibitors (Complete inhibitor cocktail, Roche; one tablet per 50 ml), and 50 mM NaF. For the Western blots, samples containing 100 or 150 μg total tissue protein from pineal glands and retinas, respectively, were loaded on the gel. The far right lane (IVT) contains *in vitro* translated rhesus macaque AANAT (2 μl). The blots were immunodetected using affinity-purified antiserum (3236) against human peptide $hAANAT_{1–26}$, which indicates total AANAT protein, and using affinity-purified antiserum (3352) against rat phospho-$rAANAT_{22–37}$, which indicates AANAT protein phosphorylated at the N-terminal PKA site. The IVT AANAT was detected by antiserum 3352. AANAT activity was measured using a radiochemical method; serum melatonin was measured by RIA.

experimental animals (3). In the present study there was a close correlation between the amount of total AANAT protein and AANAT enzyme activity measured in individual pineal glands and in retinas ($r^2 = 0.99$; n = 16). The level of pineal AANAT activity was similar to that reported for fetal rhesus macaque (25). Phosphorylated AANAT protein (N-terminal PKA site) also increased at night in both the pineal gland and retina (Fig. 3). Pituitary AANAT activity was very low, and this may have been due to acetylation of tryptamine by ANAT, a widely distributed acetyltransferase with low affinity for substrates of AANAT (26). ANAT was detectable in all tissues examined (Table 1). A day/night difference in ANAT was not observed in the pineal gland or retina, but did occur in the pituitary; the significance of this rhythm is not clear.

rhHIOMT. Pineal HIOMT activity was somewhat higher in the rhesus macaque compared with that reported for human pineal glands (9.2 *vs.* 3–4.3 nmol/h·mg protein) (17, 25, 27). Day and night pineal rhHIOMT activity and mRNA values were similar (Table 1); in addition, there was no apparent day/night pattern in retinal HIOMT mRNA within those retinal samples in which rhHIOMT was detectable (data not shown). HIOMT activity was not detectable in samples of rhesus macaque retina, as reported for human retina (19).

Localization of AANAT mRNA in the monkey pineal and retina. *In situ* hybridization revealed that rhAANAT mRNA was present in many cells uniformly distributed throughout the pineal gland (Fig. 4A). The distribution of rhAANAT mRNA in the retina was primarily limited to the photoreceptor layer; labeling of cells in the outer region of the inner nuclear layer

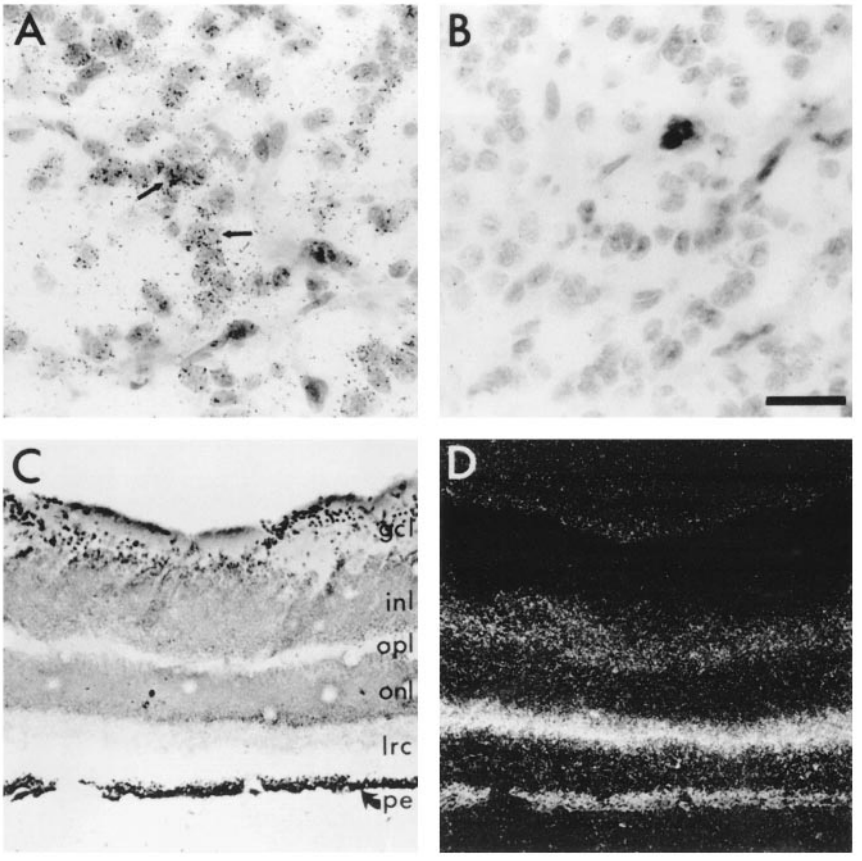

FIG. 4. Localization of AANAT mRNA in rhesus macaque pineal gland and retina by *in situ* hybridization histochemistry. A, Pineal section hybridized with an antisense riboprobe for AANAT shows grains above pinealocytes (*arrows*). B, Pineal section hybridized with a sense riboprobe for AANAT. *Bar*, 10 μm. C, Retinal section hybridized with an antisense riboprobe for AANAT and viewed under brightfield illumination. D, The same retinal section as in C, but viewed under darkfield illumination. The pigmented epithelium is not labeled, but reflects the light under darkfield illumination in both antisense and sense preparations. gcl, Ganglia cell layer; inl, inner nuclear layer; opl, outer plexiform layer; onl, outer nuclear layer; lrc, layer of rods and cones; pe, pigmented epithelium.

was also evident (Fig. 4, C and D). Expression of AANAT mRNA was not detected in the pituitary or brain by this method.

Discussion

Several notable observations were made in this study regarding the enzymes involved in the conversion of serotonin to melatonin, including the lack of a day/night rhythm in rhAANAT mRNA in the retina and pineal, the distribution of rhAANAT in the retina, and the apparent absence of HIOMT from the retina. These will be discussed sequentially below. The finding that pineal rhAANAT mRNA does not exhibit a day/night rhythm in the pineal gland indicates that regulation of melatonin production in primates is similar to that in ungulates, where mRNA increases less than 1.5-fold at night. This difference indicates that large changes in AANAT protein and activity are not generated by changes in AANAT mRNA in primates; rather, it would appear that AANAT is continually produced, and regulation occurs by cAMP inhibition of degradation by proteasomal proteolysis (28). This proteolysis-based mode of regulation is of functional importance, because it allows for rapid changes in AANAT protein, AANAT activity, and melatonin production without requiring changes in gene expression, *i.e.* the system is always "ready to go." This contrasts with the system in rodents, which requires new gene expression for AANAT activity to increase. It seems reasonable to assume that the mode of regulation found in the rhesus macaque operates in humans. Regulation of melatonin production primarily via modulation of AANAT protein appears to explain why circulating levels of melatonin increase immediately after lights-off at night in sheep, rhesus macaques, and humans (2, 6, 7, 14).

A second notable finding described here is the distinct expression of AANAT mRNA in cells in the outer region of the inner nuclear layer in addition to strong expression in the photoreceptor layer that has been previously described (10, 29). Expression of this nature, outside the photoreceptor layer, has also been reported in other species. In the case of the chicken, this occurs in the ganglia cell layer; that in the ovine and rhesus retina occurs in the outer region of the inner nuclear layer (30) (Coon, S. L., S. McCune, and D. C. Klein, unpublished observations).

The functional significance of extra-photoreceptor layer expression of AANAT is unclear. These cells might be linked to the circadian visual system, which has an extra-photoreceptor layer distribution (30–32). In rodents these cells are in the ganglia cell layer; their location in the primate retina is not known.

The third observation of interest is that HIOMT activity and mRNA are either very low or undetectable in the rhesus retina. A generally similar situation exists in the human retina: HIOMT mRNA can be detected consistently using only very sensitive methods (Southern blot of PCR products), and HIOMT activity is undetectable (16, 19). The very low expression of HIOMT in the rhesus retina supports the in-

terpretation that melatonin does not play a paracrine role in this tissue, and that pineal-derived melatonin may influence retinal function.

The high level of expression of rhAANAT in the retina is worthy of special comment. As indicated above, the level of rhAANAT mRNA is twice that in the pineal gland relative to total RNA; expression is occurring primarily in the photoreceptors cells, which make up only a fraction of the total RNA, indicating that expression in these cells may be 5- to 10-fold greater than that in the pinealocytes.

This leads to the question of the functional role that AANAT plays in these cells. Although AANAT may not be involved in melatonin production, because HIOMT appears to be absent, other roles should be considered. One is to minimize oxidative formation of aldehydes of serotonin and other arylalkylamines, including tryptamine, tyramine, and phenylethylamine. This would occur if serotonin concentrations are decreased by acetylation. In this regard, ANAT might act in parallel with AANAT (33). Aldehydes are recognized as being nonspecifically toxic because they are highly reactive; in the context of photoreceptor physiology, they could suppress the formation of photopigments by competing with retinal for binding to opsins. It is not clear whether this should be considered a toxic reaction or might represent a mechanism to modulate phototransduction. Two other potential roles of AANAT in the retina are focused on *N*-acetylserotonin; the combination of a high concentration of AANAT and the near absence of HIOMT would enhance accumulation of *N*-acetylserotonin. *N*-Acetylserotonin could act as an antioxidant (34) or a ligand for melatonin receptors (2). Although *N*-acetylserotonin is a poor ligand for these receptors relative to melatonin, local concentrations of *N*-acetylserotonin at night might be sufficient for receptor activation.

Several observations support the view that the rhesus macaque is a good model for the study of how melatonin production is regulated in humans. As mentioned in the introduction, the dark-light transition at night is associated with an immediate increase in circulating melatonin in both rhesus macaques and humans (2, 7), and dim white light suppresses melatonin (14, 15). We can now add that the levels of key melatonin synthesis enzyme activities are generally similar, and that melatonin production appears to be limited primarily to the pineal gland. The major mechanism of regulation of melatonin production in primates appears to involve posttranscriptional control of AANAT proteolysis via the highly conserved cAMP-operated PKA/14-3-3 binding switch, based on studies of hAANAT and the structure of primate AANATs (4, 20). The available data indicate that melatonin rhythm-generating systems in species from the same mammalian orders are similar (Ungulata, cow and sheep; Rodentia, rat and mouse) (3, 35). It can be reasonably expected that the judicious use of the rhesus macaque in an effort to experimentally bridge nonprimate and primate physiology will lead to a better understanding of the physiological and pharmacological regulation of human melatonin production.

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